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Physiological response of temperature shocks in turbot and sole

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RUNNING HEAD: Undercooling of turbot and sole

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Abstract

In the present study, selected temperature drops were examined in order to investigate the effects of live chilling on stress and welfare in turbot and sole. This study demonstrated that rapid temperature drops from 11 - 12°C and 18 - 18.7°C to 4-0°C in turbot resulted higher blood sodium and potassium at 0°C (164 mmol l^{-1}, 4.4 mmol $^{-1}$) compared to 4°C (153 mmol l^{-1}, 3.1 mmol $^{-1}$) indicating osmoregulatory disturbance. A rapid temperature drop from 18°C to 0°C in Senegal sole also resulted in higher blood sodium and potassium at 0°C (164 mmol l^{-1}, 4.8 mmol $^{-1}$) compared to control group at 18°C (157 mmol l^{-1}, 3.2 mmol $^{-1}$). Based on present findings we conclude that immersion in ice water will have a negative effect on the animal osmoregulatory capacity, and we recommend that turbot and sole are stunned before slaughter.

KEYWORDS: Hypothermia, Live chilling, Temperature, Sole, Turbot

INTRODUCTION

Live chilling of fish prior to slaughter provides an effective method of cooling the fish throughout the slaughter line, and postpones the onset of rigor and the time for resolution of rigor (Skjervold, Fjæra, and Østby 1999; Skjervold et al. 2001). Such rapid decreases in temperature, commonly termed "cold shock" may however result in a number of physiological and behavioural changes in fish (Donaldson et al. 2008). According to the EU directive on the protection of animals at slaughter (European Council 2009, Directive 93/119/EC) an optimal slaughter method should render fish unconscious until death without avoidable excitement, pain or suffering prior to killing. The question is whether this is possible for fish. Van de Vis et al. (2003) evaluated different industrial and research based slaughter methods for Atlantic salmon (*Salmo salar*) and classified traditional commercial slaughter methods not to be in conformity with the above directive or inconclusive. Newer methods (in use today), such as percussive and electrical stunning (Lambooij et al. 2010) were evaluated positively, but the use of live chilling prior to the slaughtering procedure was not evaluated with respect to animal welfare. An alternative to an instantaneous stun using electricity can be the application of controlled exposure to a sudden and strong temperature drop, combined with exsanguination as a killing method. The rationale for this is that fish may tolerate a well-controlled drop in temperature (Donaldson et al. 2008). Hence, in a report for the European Food Safety Authority (EFSA 2009) it is stated that "the temperature tolerance limits of turbot with regard to pre-slaughter live chilling are not clearly understood and need investigation". Thus, research is needed to establish which level of temperature decrease that can be tolerated by the fish species, taking into account the temperature at which the animal is reared.

The experiments, examining different ΔTs as well as the potentially stressful process of physical handling, were designed to investigate the effects of live chilling on physiological tolerance measured by blood parameters in turbot (*Scophthalmus maximus*) and Senegal sole (*Solea senegalensis)*. Turbot and Senegal sole are currently the flatfish species with largest rearing volume in Europe (Imsland 2010) and both are currently being slaughtered with the use of live chilling. However, these two species have different temperature optimum range in their on-growing (> 100 g) phase. For Senegal sole the optimum temperature range is 22-27°C (Dinis et al. 1999; Imsland et al. 2004) whereas for turbot the same optimum range is between 16-17°C (Irwin et al. 1999). The farming range of turbot is between 11-20°C (Imsland 2010) whereas similar range for Senegal sole is higher (up to 25°C, Imsland et al. 2004). So the physiological range of the two species is different and it would be difficult to compare their

physiological range under exact same temperature conditions. Accordingly, the aim of the present study was not to make inter-species comparison but to look at their respective physiological response of abrupt temperature changes.

We characterized the rate and magnitude of the adaptive stress response and its relationship to the welfare of the fish by measuring several well-established primary and secondary stress parameters (cortisol, glucose, lactate and ions) in blood plasma. The aim of the study is investigate if the alternative slaughter methods (electrical, live chill) improve the welfare of the fish by making it less susceptible to suffering.

MATERIALS AND METHODS

Turbot – trial 1

Juvenile turbot from a commercial producer in the Netherlands (Seafarm B.V, Kamperland, The Netherland) of 300 g on average were transported to the experimental facilities of IMARES in Yerseke (The Netherlands) in January 2013. The fish were acclimated for a period of three weeks to two temperatures (12 and 18.7ºC, termed "winter" and "summer" temperatures) in two separate 360 l rearing tanks (tank dimensions: 0.5 x 1 x 1 m). The fish were maintained under a natural photoperiod (12L:12D) and were fed with a commercial formulated feed (Efico Sigma 870 F Biomar), containing 54% protein and 14% fat (pellet-size 6.5 mm), using automatic feeders.

Mean weight (SD) of the turbot subjected to chilling was 400 (101) and 361 (38) g at 18.7 and 12ºC respectively. The fish were starved for 48 h prior to the experiment. At experimental start-up, on 18 March, fish were transferred directly into a 50 l tank using a dip net (identical light conditions as above) with seawater and melting flake ice (0°C). A total of six fish were transferred from each of the two rearing temperatures, and treatments were replicated (3 fish transferred each time). Blood samples were collected from control fish directly from the holding tank and in live-chilled fish 1 h post transfer (see below for procedures). The resulting experimental set-up consisted therefore of four groups i.e. 12°C control; 18.7°C control; 12°C-0°C; 18.7°C-0°C.

Turbot – trial 2

This trial was a refinement of the first trial where we wanted to look at more temperature drops than in the first trial. Juvenile turbot from a commercial producer in Norway (Stolt Sea Farm Turbot Norway, Øyestranda, Norway) of 250-300 g on average were transported to the experimental facilities of Akvaplan-niva in Tromsø, Norway in July 2013. The fish were acclimated over a period of 1 month to two temperatures (11 and 18ºC) in four 250 l rearing tanks. The tanks were supplied with water from a flow-through system where seawater was pumped from 60 meters depth, UV treated, particle filtered and aerated before entering the tanks. The fish were maintained under a natural photoperiod for Tromsø (24-18 h of light during July and August) and were fed with a commercial formulated feed (Amber Neptun ST, Skretting, Norway, pellet-size 2 mm), using automatic feeders (Billund Aquakulturservice,

Denmark). Mean weight (SD) of the experimental fish after the acclimation period was 280 (32) g. The fish were starved for 48 h prior to the experiment. At experimental start-up, on 22th of August, fish were transferred from the rearing tanks into two 50 l tanks using a dip net (identical light conditions as above) with ice slurry, holding water temperatures of 4 and 0ºC. A total of four fish were sampled for each transfer (six transfers in total), including blood samples for control fish. Blood samples were collected 1h post transfer (see below for procedures). The resulting experimental set-up consisted therefore of six groups i.e. 11°C control; 18°C control; 11°C-4°C; 18°C-4°C; 11°C-0°C; 18°C-0°C.

Trial with Sole

The experiment on Senegal sole took place in a commercial facility in Northern Portugal (Aquacria Piscicolas, Torreira, Portugal) in September 2013. The physiological effects of rapid temperature decrease (live chilling) and electrical stunning, as novel slaughter procedure for sole, were compared to present commercial practice (exsanguination and asphyxia on ice). Six slaughter sized sole (400-700 g) were transferred directly from a commercial holding tank (18°C) into ice slurry with a temperature of 0°C using a dip net (live chilling group). Six fish were kept at 18°C as controls. Another six fish were exsanguinated and transported to a live-chilling tank (commercial group). The forth-experimental group (direct stun) consisted of six soles netted from the holding tank and transferred into a prototype electrical stunner for flatfish. The resulting experimental set-up consisted therefore of four groups i.e. 18°C control; 18°C-0°C (live chilling); exsanguination and asphyxia on ice (commercial group); electrical stunning (direct stun group).

For electrical stunning of sole Seaside A/S (Stranda, Norway) designed and build a first prototype stunner, which was equipped with 8 rows of above-suspended positive electrodes spaced 7 cm apart. Each row consisted of 7 stainless steel electrodes of 5 cm and 25 cm length, which were the positive electrodes. The distance between these electrodes and conveyer, which is the negative electrode, was 2 cm. The experimental unit was connected to a coupled direct (DC) and alternating (AC) power source providing DC current ripped with a 100 Hz AC current on top (Llonch et al. 2012).

Blood samples from all four experimental groups were collected 1 h post transfer. Procedures were otherwise identical to those described for turbot.

Blood analyses

In all experiments, following exposure, fish were rapidly removed from the tanks and immediately killed with a sharp blow to the head. Blood was extracted from the caudal vessels using heparinized syringes, and immediately analyzed using an i-STAT Portable Clinical Analyzer (Emergo Europe, The Netherlands). The analyzer was used in conjunction with CG8+ and CG4+ disposable cartridges, measuring blood sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) content, pH, lactate and glucose levels. pH values were adjusted according to the temperature difference between 37°C and the temperature of the fish (*T*) (Ashwood et al. 1983; Merkin et al. 2010; Hosfeld et al. 2010).

Cortisol

Cortisol was measured as previously described by Gorissen and colleagues (Gorissen et al. 2012). In short, 96-well microtiter plates were coated with mouse cortisol antibodies in a coating buffer. Plates were cleared of coating buffer and washed with a wash buffer before blocking possible a-specific binding sites with a blocking buffer. Wells were cleared of blocking buffer and 10 µl of standard or sample, together with 90 µl of tracer, was added to the proper wells. After the incubation period wells were cleared and washed before scintillation liquid was added. Activity within the wells was measured using a β-counter. Due to practical limitations it was only possible to analyze cortisol in turbot in trial 1.

Statistical analysis

All statistical analyses were performed using STATISTICA^{TM} 12. To assess normality of distributions a Kolmogorov-Smirnov test (Zar 1996) was used and homogeneity of variances was tested using Levene's F test (Brown & Forsythe 1974). Analysis of blood parameter values were conducted using a two-way nested analysis of covariance (ANCOVA), using fish weight as a covariate. Significant ANCOVA's were followed by a Student-Newman-Keuls (SNK) multiple comparison test to identify differences among treatments. A significance level (α) of 0.05 was used if not stated otherwise.

RESULTS

Trial 1 – Turbot

Direct transfer, i.e. temperature drop, to a new tank resulted in significant increases in plasma levels of Na^+, K^+ and Ca^{2+} at both temperatures, compared to pre-transfer levels. The increase was most pronounced, and significant for all three parameters, in the 18.7°C group (SNK test, $P < 0.05$, Fig. 1).

In the 12°C group, a significant increase in pH was seen 1 h post-transfer to 0°C, whereas fish in the 18.7°C group did not display a similar increase. For the other parameters, lactate and cortisol, there was no significant effect from exposing the fish to rapid temperature drops (Fig. 2).

Trial 2 – turbot

A rapid temperature drop to 0°C resulted in an increase in plasma levels of Na^+ and K^+, but when the drop was less pronounced, i.e. to 4°C, there was no significant increase in either of these two parameters (Fig. 3). For all other parameters measured (pH, Glu, Ca^{2+}), no significant differences were seen between treatments.

Trial with Sole

A significant increase in Na^+ and K^+ was seen for all treatments compared to the control levels (Fig. 4). For Na^+, commercial practice resulted in significantly higher levels compared to all other treatments. Commercial procedures also resulted in significantly higher Ca^{2+} compared to all other treatments (SNK test, $P < 0.05$, Figure 4). A slight, but significant, increase in pH was seen in live-chilled fish compared to the control fish (Fig. 4). Both glucose and lactate levels were significantly elevated in the commercially slaughtered fish group compared to control and all other treatments (Fig. 4).

DISCUSSION

These experiments demonstrated that turbot and sole respond with physiological alterations when exposed to live chilling (rapid temperature drops down to 0°C), a common slaughter method for both species. For turbot, it was evident that the less severe drop in temperature down to 4°C, did not affect the fish to any noticeable degree. These results are in line with what has previously been for Atlantic salmon (Foss et al. 2012). Although Atlantic salmon is considered a more temperature tolerant species, it was shown that 4°C was the limit of the organism's thermal range during the summer, whereas rapidly chilling down towards 0°C resulted in severe sub-lethal disturbances and mortality. The temperature drops that fish are able to cope with, or not, are most interesting from a welfare perspective, but also in relation to the cold chain during production. The motivation for industrial use of live chilling when slaughtering fish mainly relates to quality and welfare aspects during the slaughtering procedure. Live chilling prior to slaughtering will efficiently decrease temperature within the fillet and will also result in a prolonged available processing time from killing to the onset of rigor mortis, as demonstrated in Atlantic salmon (Skjervold, Fjæra, and Snipen 2002). Because of the heat exchange properties of the gills, the rate of thermal exchange is higher in living fish compared to dead fish when exposed to swift temperature changes (Stevens and Sutterlin 1976; Skjervold, Fjæra, and Østby 1999). In that aspect, live chilling can be considered positive in terms of welfare for slowing down the metabolism during transport, for better holding and live handling conditions, and to enhance an early cool chain. However, once the temperature tolerance levels for the fish are broken, a series of consequences causes failure to thrive, which includes osmoregulatory and respiratory failure followed by death (Foss et al. 2012). In addition, there are species-specific differences in the impact of temperature shock. For example, Roth, Imsland and Foss (2009) found that a thermal shock caused osmoregulatory and respiratory disturbances for turbot, but did not cause any mortality. In contrast, for sole, a temperature-drop down to 0°C had little influence on osmoregulation, whereas respiratory disturbances were observed. Whether these different effects are based on physiological differences between the species or simply size differences is uncertain.

A thermal shock is commonly used for both turbot and sole to stun and kill the animal prior to shipping or processing. Previous studies have shown that the animal will eventually lose all behavioural responses (Morzel, Sohier, and van de Vis 2003; Roth, Imsland, and Foss 2009). Thus, while fish may appear unconscious due to muscle contractions, they can recover very

quickly once thawed (Roth, Imsland, and Foss 2009). Based on present findings, stunning with a thermal shock is challenging from a welfare perspective for both species as clear indications of osmotic disturbances were found in both species following a thermal stunning in ice slurry at 0°C. In order to immobilize, stun and kill the animal, chilling must go beyond the limits for the species tolerance levels, thereby causing serious osmoregulatory and respiratory disturbances to ensure mortality. This requires time, depending on the temperature drop and size of the fish. From a welfare perspective, electrical stunning is more promising. Previous studies on a broad range of species have shown that electrical stunning can render the fish unconscious within one second (Lambooij et al. 2007, 2008, 2009, 2010, 2015). The challenge is however that the fish can recover during exsanguination (Lambooij et al. 2010). To protect the welfare of fish at slaughter, these animals should be rendered unconscious and insensible prior to killing. Furthermore, the state of unconsciousness must be long enough to allow killing without recovery. Based on present data and previous trials performed by the research group behind this study (Roth, Foss, and Imsland 2009; Daskalova et al. 2016) we recommend electronic stunning followed by immersion in ice water can be developed into an effective stunning and killing method for turbot and sole.

The measured blood plasma values are in the range found for control groups in previously published results in studies on stress physiology in turbot (lactate, cortisol, van Ham et al. 2003; Na^+, K^+, Ca^{2+}, pH and glucose; Imsland et al.. 2008; Roth, Imsland, and Foss 2009) and Senegal sole (lactate, Ribas et al. 2007; Na^+, Ca^{2+}, K^+, Perez et al. 2015). The only exception are higher lactose values found for turbot in the control groups (2.3-2.7 mmol l^{-1}) compared to those (0.5 mmol l^{-1}) of van Ham et al. (2003), However, van Ham et al. (2003) found a significant correlation between plasma lactate and size. This may help to explain the differences found in plasma lactate values as the turbot in present study were larger (280-400 g) than those in van Ham et al. (2003) study (40-100 g). The higher lactate values may reflect larger anaerobic muscle capacity in the larger fish as demonstrated in other fish species (Pottinger et al. 1998). The turbot sampled in the present trial were small in comparison with standard commercial slaughter size (1-2 kg). But comparison with blood physiology data from larger turbot (Imsland et al. 2008; Roth, Imsland, and Foss 2009) does not reveal any size dependent changes in the plasma values of Na^+, K^+, Ca^{2+}, pH and glucose.

Lambooij et al. (2015) measured the heart beat for the turbot in trial 1 and found that heart rates increased immediately after immersion in ice water and then decreased to a low basal value 30 min. after immersion and concluded that immersion in ice water may not induce unconsciousness.

Conclusions and future perspectives

The findings of this study shows that rapid temperature drops from 11 and 18°C to as low as 0°C results in osmoregulatory disturbance, whereas less pronounced drops, i.e. to 4°C, will have less impact. From these results we conclude that immersion in ice water will have a severe negative effect on the animal osmoregulatory capacity, and we strongly recommend that turbot and sole are stunned before slaughter.

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Compliance with ethical standards The work in the Netherlands (turbot – trial 1) and Portugal (trial on sole) were done in accordance with current European and Dutch regulations on the use of animals for scientific purposes (approved by the Animal Care and Use Committee). The Norwegian experiment (turbot – trial 2) described has been approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority (NARA) and registered by the Authority.

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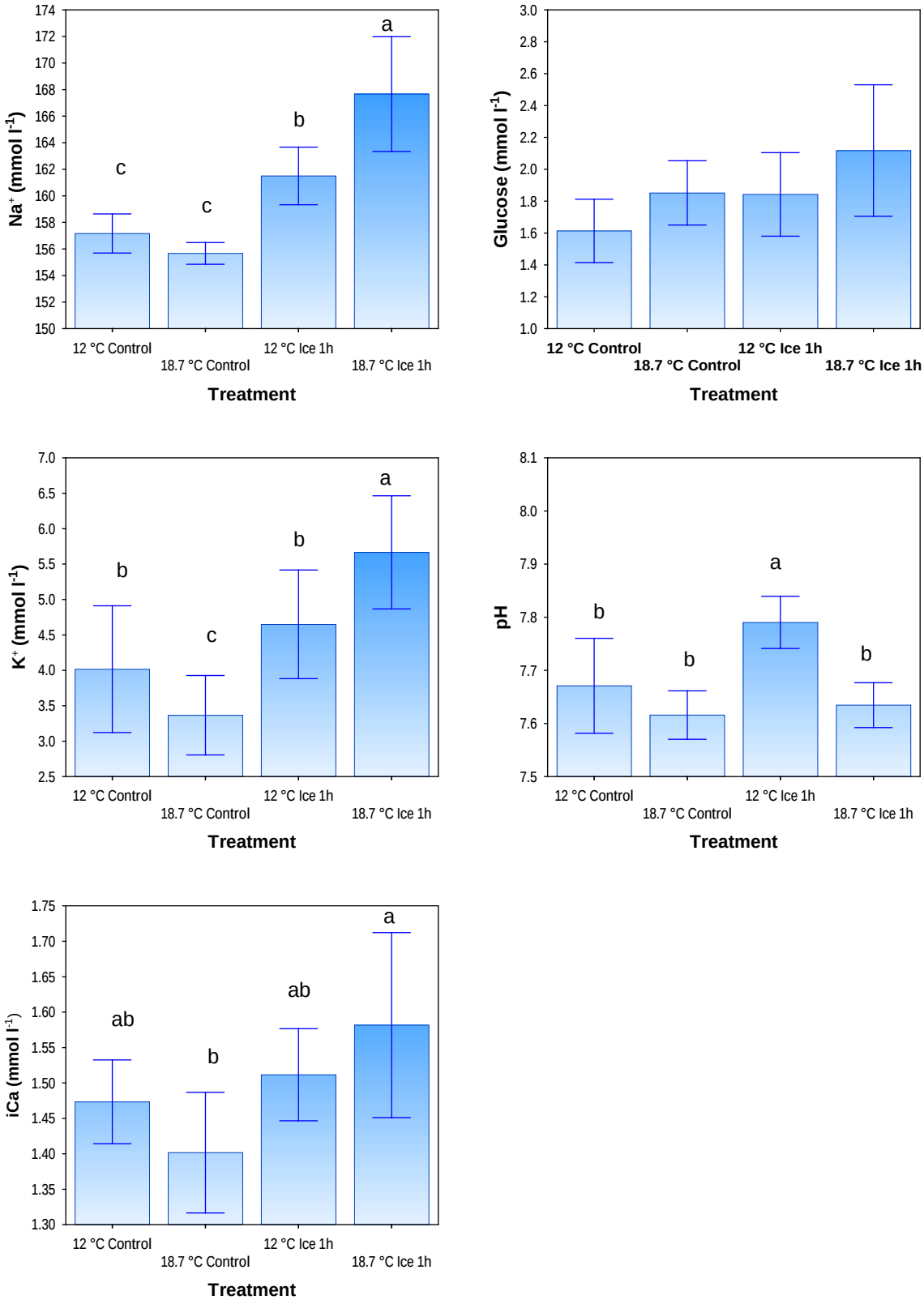
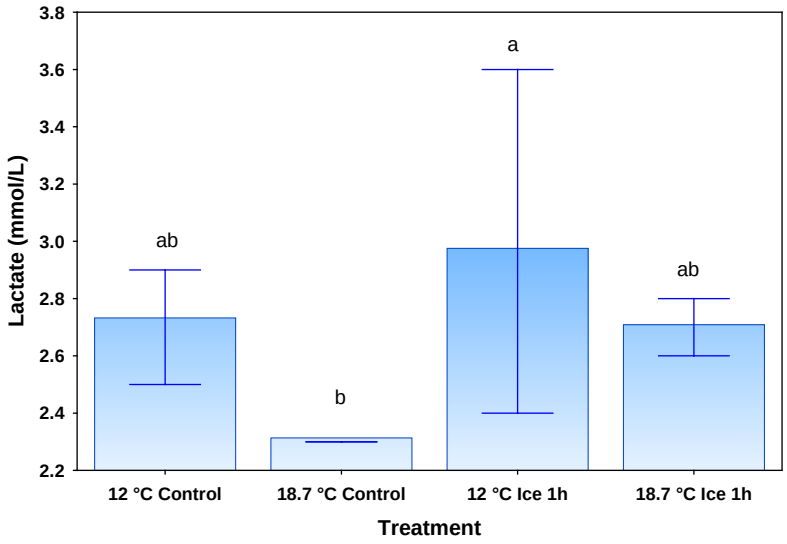


FIGURE 1 Changes in blood ions, glucose and pH in turbot acclimatized to 12 and 18.7ºC experiencing a temperature drop to 0ºC. Samples were collected 1h post temperature drop. Values are given as mean (SD). *n*=6 for all groups. Different letters denote significant difference (Student–Newman–Keuls (SNK) test, $P < 0.05$) between treatments



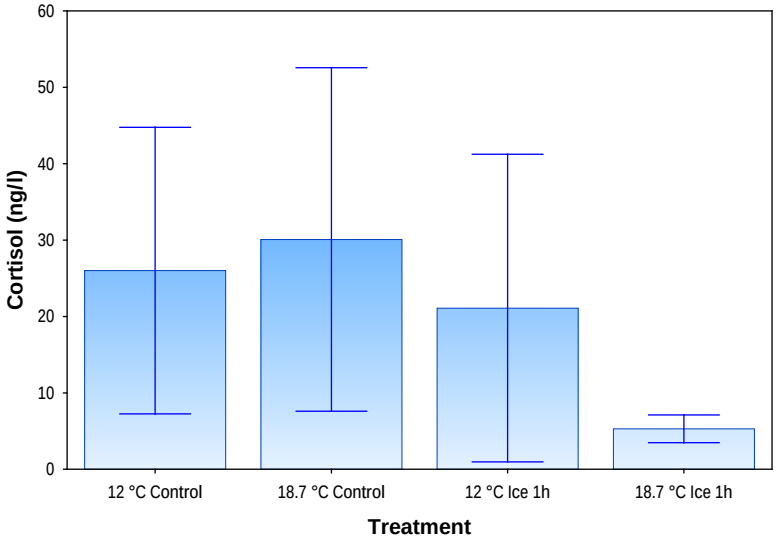


FIGURE 2 Changes in lactate and cortisol in turbot acclimatized to 12 and 18.7ºC experiencing a temperature drop to 0ºC. Samples were collected 1h post temperature drop. Values are given as mean (SE). *n*=6 for all groups. Different letters denote significant difference (SNK test, $P < 0.05$) between treatments.

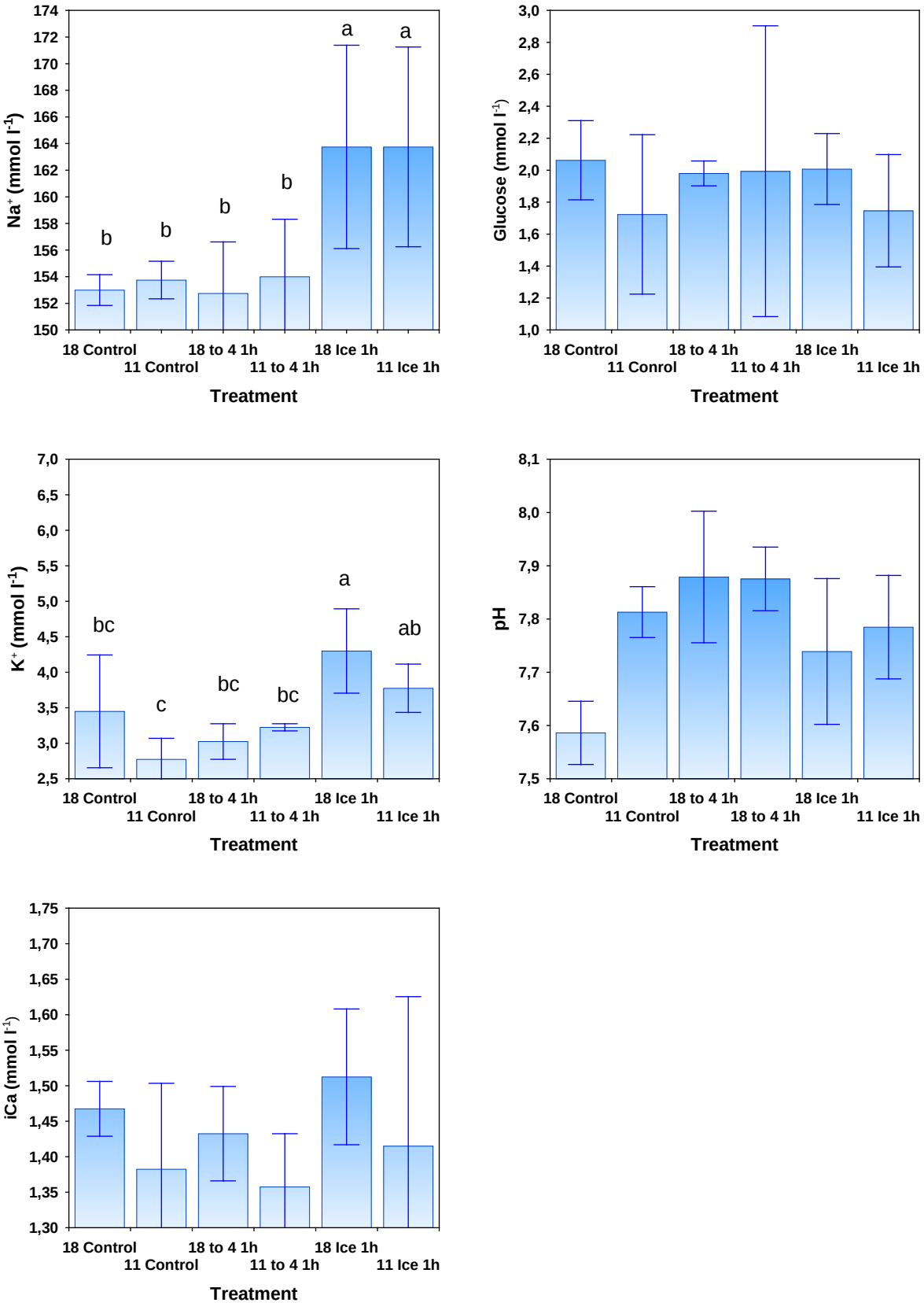


FIGURE 3 Changes in blood physiological parameters in turbot acclimatized to 18 and 11ºC and experiencing temperature drops to 4 or 0ºC. Samples were collected 1h post temperature drop. Values are given as mean (SD). *n*=6 for all groups. Different letters denote significant difference (SNK test, $P < 0.05$) between treatments.

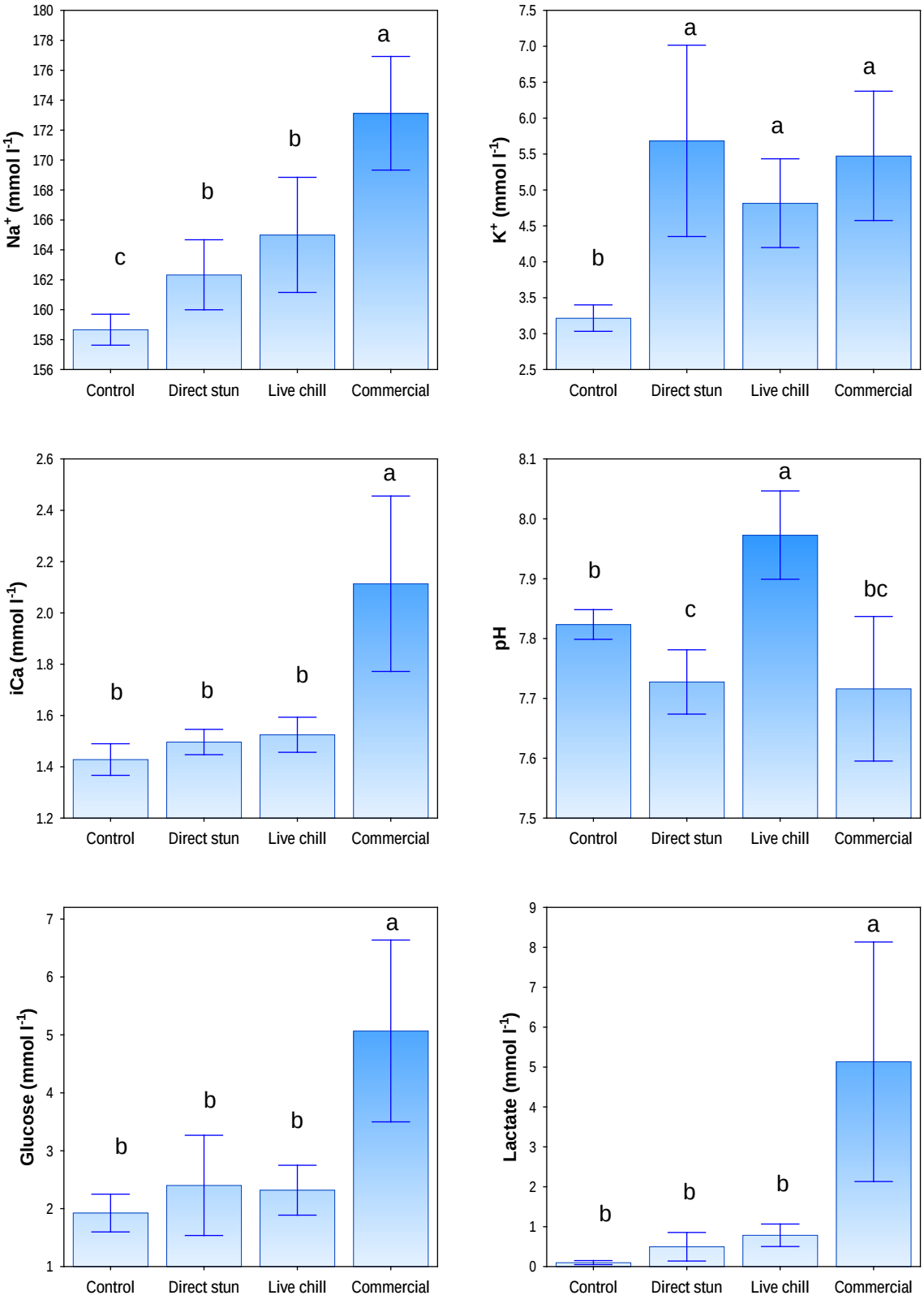


FIGURE 4 Blood ion, ph, glucose and lactate concentration in Senegal sole undergoing electrical stunning, rapid live-chilling, commercial slaughtering procedures as well as a control group (unhandled fish). Values are given as mean (SD). *n*=6 for all groups. Different letters denote significant difference (SNK test, $P < 0.05$) between treatments.